Old and new experiments for obtaining quaternary-carbon-only NMR spectra

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Abstract

In this review, several Nuclear Magnetic Resonance (NMR) pulse sequences aimed at obtaining quaternary-carbon-only (Cq only) ^{13}C spectra were evaluated both theoretically and experimentally. A new experiment, the *I*mproved *Q*uaternary *C*arbon *D*etection (*i*QCD) that improves the performances of the state-of-the-art Subsequent Editing using a Multiple Quantum Trap-90 (SEMUT-90) pulse sequence is also proposed and described. Compared to the latter, the *i*QCD sequence permits using shorter tip angles for the first carbon pulse to allow for a faster repetition rate and better sensitivity. With concentrated sample and by taking advantage of the high sensitivity of cryogenically cooled probeheads, artifacts-free quaternary-carbon-only spectra can be obtained with very few scans. These attributes make the *i*QCD NMR experiment attractive for ^{13}C analysis of mixtures of medium-sized molecules and quaternary carbon-rich synthetic products.

Keywords: NMR, ^{13}C, Cq-only spectrum, artefacts, sensitivity.

Introduction

Despite the availability of a large number of multidimensional heteronuclear NMR techniques for ^{13}C spectral assignments[1], one-dimensional spectral (1D) editing ^{13}C experiments, such a Spin-Echo Fourier Transform (SEFT)[2], Attached Proton Test (APT)[3], Polarization Enhancement Nurtured During Attached Nucleus Testing (PENDANT)[4, 5], refocused variants of Insensitive Nuclei Enhanced by Polarization Transfer (INEPT)[6], Distortionless Enhancement by Polarization Transfer (DEPT)[7, 8], Distortionless Enhancement by Polarization Transfer including Cq (DEPTQ)[9-11], and Subsequent Editing using a Multiple Quantum Trap (SEMUT)[12] continue to be very useful for routine applications of ^{13}C spectral assignments. This can be accounted for two reasons. First, it turns out that even today many chemists and natural product chemists do not always "rely upon" multinuclear 2D NMR spectra, and favor the visualization of classical 1D resonances. There are however many excellent educational reviews explaining how to choose the best 2D sequences, acquisition parameters, and processing methods.[13-15] Second, under standard experimental conditions, the resolution achieved in the indirect dimension (^{13}C) of multinuclear multidimensional experiments, like Heteronuclear Single Quantum Coherence (HSQC)[16], Heteronuclear Multiple Bond Correlation (HMBC)[17-19] or Long-Range Heteronuclear Single Quantum Multiple Bond Coherence (LR-HSQMBC)[20], is rather low. The unambiguous differentiation between all ^{13}C resonances can be therefore complicated, if not impossible, unless non-standard experiments are recorded[21, 22] or post acquisition software's to artificially enhance the resolution are used.[23] These methods have undoubtedly their merits, but can be only used to a certain extent in a routine manner.

Moreover, owing to the large number of samples, the goal of many laboratories is to provide rapid analyses with the focus on checking proposed structures rather than attempting full characterization. Thus, being cognizant of the exact number of quaternary carbons at an early stage of the structure elucidation process can be very useful. Indeed, while this parameter alone is not sufficient for determining the structure, it can allow the establishment of the general category (i.e. steroid,…) of the target molecule, and allows rejecting a large number of possible other skeletons.

1D ^{13}C experiments such as APT[2], PENDANT[4,5], DEPTQ[9-11], provide information about both quaternary and protonated carbons in which C and CH_2 carbons are 180° out-of-phase with respect to the CH and CH_3 carbons. However, it is not possible to directly distinguish between C and CH_2 carbons and between CH and CH_3 carbons from these experiments since they appear with the same phase. In theory it is possible to distinguish between C and CH_2 carbons as the resonances of quaternary carbons are normally less intense, because they benefit from weaker Nuclear Overhauser Enhancement (NOE) and usually have very long T_1 relaxation times.[24] Practically, it turns out that this distinction is not always possible and reliable. Yet, in some specialized applications, or when dealing with mixtures of compounds, scientists may be interested in obtaining ^{13}C spectra in which only the quaternary carbons are visible. As shown later, such so-called quaternary-carbon-only (Cq-only) spectra could be particularly useful for isolating cross peaks belonging exclusively to quaternary carbons in crowded HMBC or LR-HSQMBC spectra.

If the evolution delays Δ for transferring magnetization form protons to carbons (or heteronuclei) via the heteronuclear coupling is set to specific values (SEFT, APT, DEPT, Pulsed-Field-Gradient Quaternary Carbon Detection, PFG-QCD), or for specific

proton pulse lengths (SEMUT-90, SEMUT-GL-90) or for special gradient combinations (DEPTQ), Cq-only spectra can be obtained.[2, 9, 10, 12, 25-28] Such experiments appear more attractive and robust than the approach based on the subtraction of experiments recorded using different proton selection pulses for being used in a routine manner.[11, 12, 25]

In this contribution, several pulse sequences able to provide Cq-only spectra are examined both theoretically and experimentally. A slightly modified SEMUT-90 experiment, termed *I*mproved Quaternary Carbon Detection experiment (*i*QCD), able to provide clean Cq-only spectra with superior sensitivity when used in a routine manner, is also introduced and discussed.

Materials and Methods

All NMR experiments were recorded on a BRUKER AvanceII-500 spectrometer equipped with a dual-channel 5 mm inverse BBI probehead incorporating a z-gradient coil. The test sample was cholesteryl acetate (30 mg) dissolved in $CDCl_3$ (0.7 mL). All spectra were recorded using a ^{13}C spectra width of 200 ppm centered at 100 ppm using 32'768 points leading to an acquisition time of 1.3s. ^{1}H and ^{13}C 90° pulse lengths were 8.2 μs and 13.2 μs, respectively. The Broadband Universal Rotations By OPtimal control (BURBOP) pulse for ^{13}C refocusing has a duration of 2 ms, is defined by 4000 points and 50 kHz sweep width (Burbop-180.1 in the Bruker wave form library). The duration of each gradient was δ = 1 ms, the gradient recovery was 0.2 ms. All spectra were acquired using TopSpin® 3.2.6 (Bruker BioSpin) and processed using TopSpin® 4.0.8 (Bruker BioSpin). Prior to Fourier transformation, zero filling to 65'536 points and weightingwith an exponential function (Line broadening (LB) = 1 Hz) were applied.

Results and Discussion

Theoretical Background

As an example of the contrasting quality of Cq-only spectra that can be obtained using the different pulse schemes aforementioned, DEPTQ135, Cq-only DEPTQ135, PFG-QCD, SEMUT-90, SEMUT-GL-90 experiments were recorded on a sample of cholesteryl-acetate and are shown in Figure 1. Cholesteryl-acetate (Figure 2) contains four quaternary carbons, C4 at δ = 170.7 ppm, C7 at δ = 139.9 ppm, C12 at δ = 42.5 ppm and C15 at δ = 36.8 ppm (Supplementary Information). Of note, in the DEPTQ135 spectrum (Figure 1A), the resonances of C12 at δ = 42.5 ppm and C15 at δ = 36.8 ppm cannot be attributed directly, as both resonances do not appear with the typical low intensity compared to that of proton-bearing carbons. It should be mentioned here that with the evolution delays set to $\Delta = 0.5/{}^{1}J_{CH}$, the APT or SEFT pulse sequence can theoretically also provide Cq-only spectra. However, the APT sequence was not included in the comparison, as it is long known that numerous and very strong artefacts occur in the resulting Cq-only spectra.

Out of the five Cq-only spectra shown in Figure 1, the spectrum obtained with the SEMUT-90 pulse sequence, with $\Delta_1 \neq \Delta_2$ (Figure 1E) provides the best results, and is totally free of artifacts originating from CH_n groups. Moreover, SEMUT-90 also provides the best signal-to-noise ratio for those quaternary carbons. On the other hand, the Cq-only DEPTQ135 (Figure 1B), the PFG-QCD (Figure 1C), the SEMUT-90, with $\Delta_1 = \Delta_2$ (Figure 1D) pulse sequences are less sensitive and exhibit artifacts originating from CH_n groups with variable intensity. In our hands, the SEMUT-GL-90 performed similar to the simple

SEMUT-90, although it was specifically designed for very efficiently suppressing *J*-cross-talk in edited ^{13}C subspectra.[26] Another unexpected result is that the SEMUT-90 sequence always performed *worse,* considering *only* the suppression of signals from CH_n groups but *not* the sensitivity*,* when the ^{13}C 180° square pulse was replaced by a classical chirp adiabatic inversion pulse[29] or a broadband optimized BURBOP pulse.[30, 31] (Figure S2)

(insert Figure 1)

(insert Figure 2)

In Cq-only spectra, the perfect suppression of proton bearing carbons can only be achieved for ideal timing when the evolution delays $\Delta = 0.5/^1J_{CH}$, which is obviously not the case for all CH_n groups within a molecule. The product operator evaluation of the PFG-QCD pulse sequence, using the notation introduced by Valeriu and Mateescu[32], for primary, secondary, tertiary and quaternary (CH_3, CH_2, CH and C_q) carbons considering the general case $2\Delta \neq 0.5/^1J_{CH}$ demonstrates the inevitable presence of potentially very strong CH_n artefacts in a Cq-only spectrum.

<u>Cq:</u>

$$[-z] \xrightarrow{90°xC} [y] \rightarrow \xrightarrow{G1\ 180°xC\ 2\Delta\ G1} \rightarrow \rightarrow -[y] \xrightarrow{90°xC} -[z] \xrightarrow{G2} -[z] \xrightarrow{90°xC} [y] \xrightarrow{^1H Decoupling} [y] \quad (1)$$

<u>CH:</u>

$$[-z1] \xrightarrow{90°xC} [y1] \rightarrow \xrightarrow{G1}\xrightarrow{180°xC}\xrightarrow{180°xH}\rightarrow\xrightarrow{2\Delta}\rightarrow\xrightarrow{G1} -c[y1] + s[xz] \xrightarrow{90°xC} -c[z1] + s[xz] \xrightarrow{G2}\rightarrow$$

$$-c[z1] \xrightarrow{90°xC} c[y1] \xrightarrow{^1HDecoupling} c[y1]$$

(2)

With c = cos($\pi^1 J_{CH} 2\Delta$), s = sin($\pi^1 J_{CH} 2\Delta$)

<u>CH_2:</u>

$$[-z11] \xrightarrow{90°xC} [y11] \rightarrow \xrightarrow{G1}\xrightarrow{180°xC}\xrightarrow{180°xH}\xrightarrow{180°xH'}\xrightarrow{2\Delta}\rightarrow\xrightarrow{G1} -c^2[y11] + cs[x1z] + cs[xz1]$$

$$+ s^2[yzz] \xrightarrow{90°xC} -c^2[z11] + cs[x1z] + cs[xz1] + s^2[zzz] \xrightarrow{G2}\rightarrow -c^2[z11]$$

$$+ s^2[zzz] \xrightarrow{90°xC} c^2[y11] - s^2[yzz] \xrightarrow{^1HDecoupling} c^2[y11]$$

(3)

With $c^2 = \cos^2(\pi^1 J_{CH} 2\Delta)$, $s^2 = \sin^2(\pi^1 J_{CH} 2\Delta)$

<u>CH_3:</u>

$$[-z111] \xrightarrow{90°xC} [y111] \rightarrow \xrightarrow{G1}\xrightarrow{180°xC}\xrightarrow{180°xH}\xrightarrow{180°xH'}\xrightarrow{180°xH''}\xrightarrow{2\Delta}\rightarrow\xrightarrow{G1}\xrightarrow{90°xC}\xrightarrow{G2}\rightarrow -c^3[z111]$$

$$+ cs^2[z1zz] + cs^2[zz1z] + cs^2[zzz1] \xrightarrow{90°xC} c^3[y111] - cs^2[y1zz]$$

$$- cs^2[yz1z] - cs^2[yzz1] \xrightarrow{^1HDecoupling} c^3[y111]$$

(4)

With $c^3 = \cos^3(\pi^1 J_{CH} 2\Delta)$

A similar product operator evaluation of the SEMUT-90 pulse sequence considering the general case $\Delta \neq 0.5/^1J_{CH}$ shows that the SEMUT-90 has an inherent predisposition to better suppress the signals of CH_n groups in a Cq only spectrum.[12, 26]

Cq:

$$[z] \xrightarrow{90°xC} -[y] \xrightarrow{180°xC\ 90°xH\ 2\Delta} [y] \xrightarrow{1_{HDecoupling}} [y]$$

(5)

CH:

$$[z1] \xrightarrow{90°xC} -[y1] \xrightarrow{180°xC\ 90°xH\ \Delta} c[y1] - s[xy] \xrightarrow{\Delta} c^2[y1] - sc[xz]$$

$$- s[xy] \xrightarrow{1_{HDecoupling}} c^2[y1]$$

(6)

CH_2:

$$[z11] \xrightarrow{90°xC} -[y11] \xrightarrow{180°xC\ 90°xH\ 90°xH'\ 2\Delta\ 1_{HDecoupling}} c^4[y11]$$

(7)

CH_3:

$$[z111] \xrightarrow{90°xC} -[y111] \xrightarrow{180°xC\ 90°xH\ 90°xH'\ 90°xH''\ 2\Delta\ 1_{HDecoupling}} c^6[y111]$$

(8)

The product operator evaluation of the DEPTQ pulse sequence is much more complicated.[8, 33] The final results for θ = 90° are provided below:

Cq:

$$[z] \xrightarrow{DEPTQ} [x]$$

(9)

CH:

$$[z1] \xrightarrow{DEPTQ} -c^4[x1]$$

(10)

CH_2:

$$[z11] \xrightarrow{DEPTQ} -c^8[x11] - c^4 s^4[x11] - 2c^3 s^3 s'2[y11]$$

(11)

With s'2 = $\sin(\pi^n J_{HH} \cdot 2\Delta)$

CH_3:

$$[z111] \xrightarrow{DEPTQ} very\ long\ expression$$

(12)

In Figures S3 and S4, the theoretical intensities expressed in percentage of a CH group as a function of the $^1J_{CH}$ showing up in a PFG-QCD and SEMUT-90 spectra are shown. Two cases were considered: (i) a standard $^1J_{CH}$ coupling range 120 – 170 Hz that can be used for most of the molecules, and (ii) the full $^1J_{CH}$ coupling range 110 – 250 Hz for molecules including for instance furanyl or acetynly moieties. Clearly, the SEMUT-90 performed much better and has the capability to provide ultra clean Cq-only spectra than for instance the PFG-QCD experiment. The same conclusions can be drawn for CH_2 groups (110 Hz < $^1J_{CH}$ < 150 Hz, Figures S5 and S6) and CH_3 groups (100 Hz < $^1J_{CH}$ < 130 Hz, Figures S7 and S8). However, as mentioned in the preceding paragraphs, when the first ^{13}C pulse was set to angles larger than 90° in SEMUT-90 experiments to allow

for a faster repetition rate, intense artifacts originating from CH_n groups were present in the spectra (Figure S1).

The iQCD pulse sequence

Quaternary carbons typically have very long T_1 longitudinal relaxation times (up to 100 s), thus maximizing their signal-to-noise ratio requires using excitation pulses < 90° following the Ernst angle strategy.[34, 35] When SEMUT-90 experiments were recorded by setting the first ^{13}C pulse larger than 90° to allow for a faster repetition rate (note that the first ^{13}C pulse must be larger than 90° in SEMUT, otherwise the magnetization not detected is aligned along the *–z* axis before acquisition and is therefore *not* available for the next scan), it turned out that the experiment was not performing satisfactorily, as intense artifacts originating from CH_n groups were present in the spectra (Figure S1).

A new experiment, *i*QCD (*I*mproved Quaternary Carbon Detection), which is derived from the SEMUT-90 pulse sequence was thus designed. The *i*QCD provides identical results compared to SEMUT-90, but shows better performance when used with faster repetition rates and Ernst angles. Both pulse sequences are shown in Figure 3. Compared to the SEMUT-90 experiment, the *i*QCD pulse sequence consists in two consecutive spin echoes periods, (Δ-180°$_x$(^{1}H),180° $_x$ (^{13}C)-Δ), of length $0.5/J_{CHmin}$ and $0.5/J_{CHmax,}$ respectively. As in the SEMUT-90, the 90°$_x$(^{1}H) pulse inserted between the two spin echoes transforms part of the magnetization in double quantum terms that do not evolve under the $^1J_{CH}$ coupling during the second spin echo and are eliminated.[36] As such, the product operator evaluation of the *i*QCD pulse sequence provides exactly the same outcome than the evaluation provided above for the SEMUT-90 experiment (Equations

5-9). Yet, due to the presence of two 180° ^{13}C pulses, the first ^{13}C pulse can be adjusted to the Ernst angle for maximizing the sensitivity, a strategy already used since the early 80s in the APT experiment.[3] As experimentally evidenced for the SEMUT-90 experiment,[26] a much better suppression of CH_n artefacts is obtained when the two spin echoes are set to different values: $\Delta_1 = 0.5/(J_{min} + 0.146(J_{max} - J_{min}))$; $\Delta_2 = 0.5/(J_{max} - 0.146(J_{max} - J_{min}))$ following the strategies used for low-pass *J* filters (LPJFs) in HMBC experiments.[21, 37] Finally, as it is likely that the *i*QCD experiment will also be used with automatic sample changers, a basic phase cycle to reduce the effects of possibly miscalibrated pulses was implemented.

(insert Figure 3)

From equations 5-9 and Figures S3-S8 it is clear that the degree of CH_n suppression achieved in the Cq-only spectrum using the *i*QCD experiment described is fully sufficient for routine purpose.

Experimental

The *i*QCD experiment was tested on cholesteryl acetate and compared to the SEMUT-90 experiment (Figure 4). The spectra exemplify the capability of the new *i*QCD pulse sequence to efficiently remove the signals originating from protonated carbons and provide clean Cq-only spectra, comparable to the SEMUT-90 experiment.

(insert Figure 4)

If one is interested in recording such Cq-only spectra, the main objective is to maximize the signal-to-noise ratio for a given measurement time. Because of the usually long T_1 relaxation times of quaternary carbons, maximizing their signal-to-noise ratio requires using ^{13}C excitation pulses with a pulse width less than 90° following the Ernst angle strategy.[34, 35] Unlike the SEMUT-90 experiment (Figure S1), the proposed *i*QCD sequence performs well when ^{13}C excitation pulses less than 90° are used (Figure 5). Interestingly, the quality of the suppression of the signals originating from CH_n groups is also improved when tip angles less than 90° are used, particularly visible in the spectrum recorded with a ^{13}C excitation pulse with a pulse width of 30° (Figure 5, top).

(insert Figure 5)

Cholesteryl acetate is a mid-sized organic molecule (MW = 428.69) which represents the typical molecular size for which the proposed *i*QCD experiment appears ideal. The optimal conditions for obtaining the highest signal-to-noise ratio for a given measurement time were therefore investigated. To this end, four different *i*QCD experiments with a total experimental time of 22 min were recorded: the total acquisition time was 1.3s, and the following parameters were used: (a) relaxation delay of 4 s, first ^{13}C pulse of 90° and 256 scans, (b) relaxation delay of 3 s, first ^{13}C pulse of 75° and 320 scans, (c) relaxation delay of 2 s, first ^{13}C pulse of 60° and 416 scans, and (d) relaxation delay of 1 s, first ^{13}C pulse of 30° and 592 scans (Figure S7). The results are summarized in Figure 6. It can be seen that both combinations relaxation delay of 3 s / first ^{13}C pulse

of 75° (grey) and relaxation delay of 2 s / ^{13}C pulse of 60° (medium grey) provide the overall best signal-to-noise ratio. An exception is the resonance of $CDCl_3$, which is representative of a quaternary carbon with a very long T_1 relaxation time, as for instance in acetylenyl moieties. For this carbon, obviously, the setting relaxation delay of 1s / first ^{13}C pulse of 30° provides the best signal-to-noise ratio, as the Ernst angle for these specific quaternary carbons is very small.[35] For routine applications and mid-sized molecules, the use of a relaxation delay of ~2 s and a first ^{13}C pulse θ of ~60° is advocated. This setting will provide an optimum signal-to-noise ratio for quaternary carbons exhibiting short to medium longitudinal relaxation times T_1, while keeping the overall experimental time reasonable.

(insert Figure 6)

Different *i*QCD experiments with a constant number of scans (256) were also recorded: (a) relaxation delay of 4 s, first ^{13}C pulse of 90° and total experimental time of 22 min, (b) relaxation delay of 3 s, first ^{13}C pulse of 75° and total experimental time of 18 min, (c) relaxation delay of 2 s, first ^{13}C pulse of 60° and total experimental time of 13.5 min, and (d) relaxation delay of 1 s, first ^{13}C pulse of 30° and total experimental time of 9.5 min. The results are summarized in Figure 7. In this case, the combination relaxation delay of 4 s / first ^{13}C pulse of 90° provides the overall best signal-to-noise ratio, followed by the combination relaxation delay 3 s / first ^{13}C pulse of 75°, relaxation delay of 2 s / first ^{13}C pulse of 60° and relaxation delay of 1s / first ^{13}C pulse of 30°, highlighting that the Ernst angle for typical quaternary carbons is relatively close to 90° for relaxation delays less than 5 s. An exception is again the resonance of $CDCl_3$, a

quaternary carbon with a very long T_1 relaxation time. For this carbon, the experimental signal-to-noise ratio is nearly identical, irrespective of the combination relaxation delay / length of the ^{13}C pulse, highlighting again that the Ernst angle for these specific quaternary carbons is very small for relaxation delays less than 5 s.

(insert Figure 7)

Anticipating that the *i*QCD experiment will also be used with automatic sample changers, its robustness towards miscalibrated pulses was tested. In Figure 8, an *i*QCD spectrum with perfectly calibrated ^{1}H and ^{13}C pulses is shown together with an *i*QCD spectrum recorded with the ^{1}H and ^{13}C pulses deliberately lengthened by +20%, which would mimic a sample dissolved in a solution containing > 100 mmol of ions. It can be appreciated that the intensity of the artifacts remains very low, even when run under very harsh conditions.

(insert Figure 8)

Another potentially interesting utility of Cq-only experiments is to use the resulting spectra as F_1 projection in HMBC or LR-HSQMBC spectra. As seen in Figure 9, set as F_1 projection, 1D ^{13}C *i*QCD spectra also help easily identifying cross peaks that belong to quaternary carbons. If the standard DEPTQ or APT spectra are set as F_1 projections, the identification of cross peaks that belong to quaternary carbons is difficult, and requires an almost complete analysis and attribution of all the carbons of the molecule considered.

(insert Figure 9)

Conclusion

In this work, we have presented and evaluated several pulse sequences dedicated to provide Cq-only ^{13}C spectra. It turns out that the SEMUT-90 pulse sequence provides the best results; not only is the degree of suppression of CH_n artifacts the highest, but also the overall sensitivity of the quaternary carbons is the highest. Yet, the SEMUT-90 experiment does not perform satisfactorily when recorded with the angle of the first ^{13}C excitation pulse is larger than 90° to allow for a faster repetition rate. The proposed *i*QCD experiment presented in this work remedies to this problem, and yields Cq-only spectra with an identical degree of suppression of CH_n artifacts, but yield clean Cq-only spectra with higher signal-to-noise ratios when ^{13}C excitation pulses shorter than 90° are used. In addition, the *i*QCD experiment works very well even when the proton and/or carbon pulses are miscalibrated, and is therefore inclined to be used with automatic sample changers. These attributes make the *i*QCD NMR experiment attractive as an additional tool focused on quaternary carbons, particularly in high-throughput analysis laboratories or under automation, for rapid analysis of newly synthesized or extracted medium-size molecules, typically natural products.

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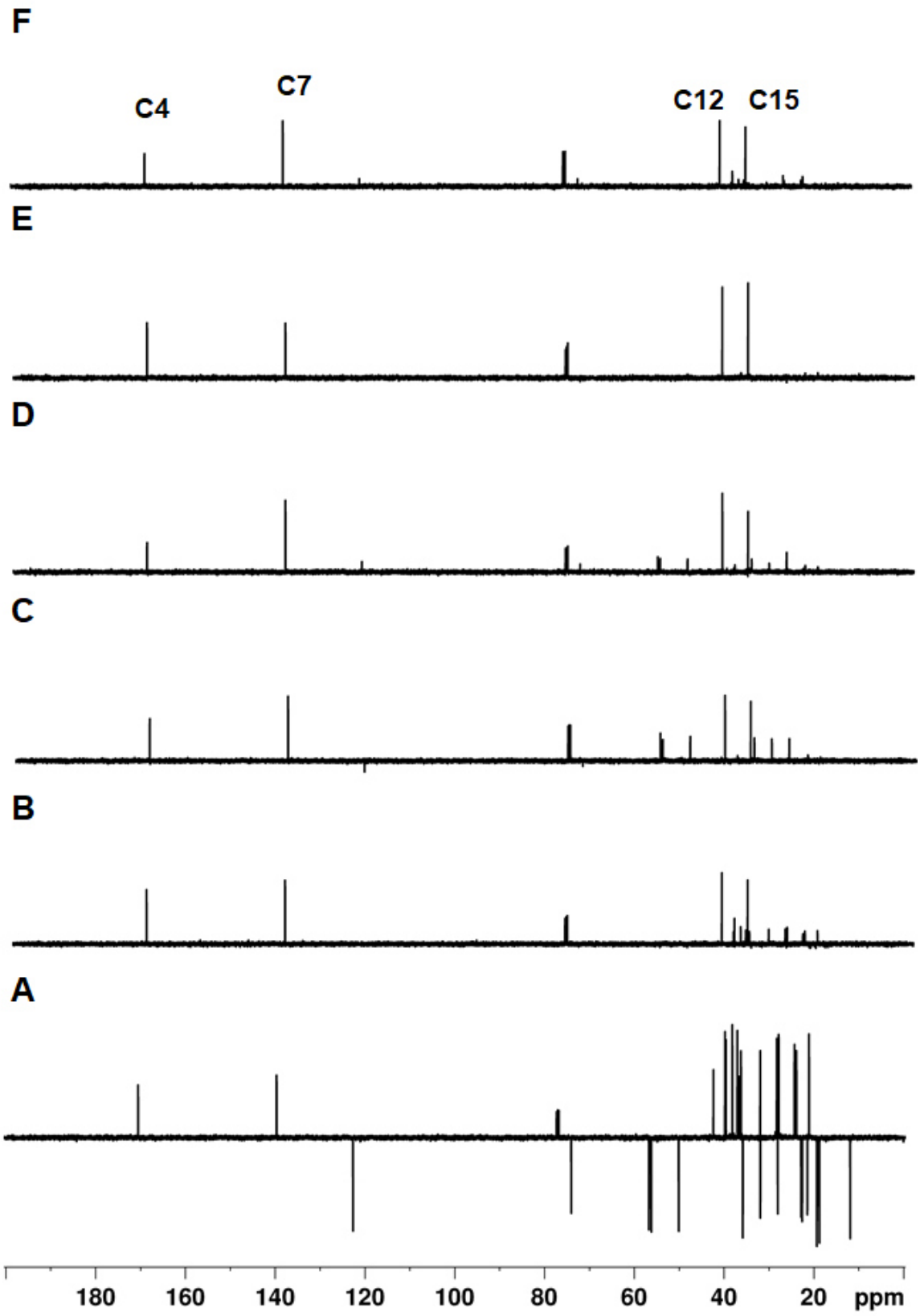

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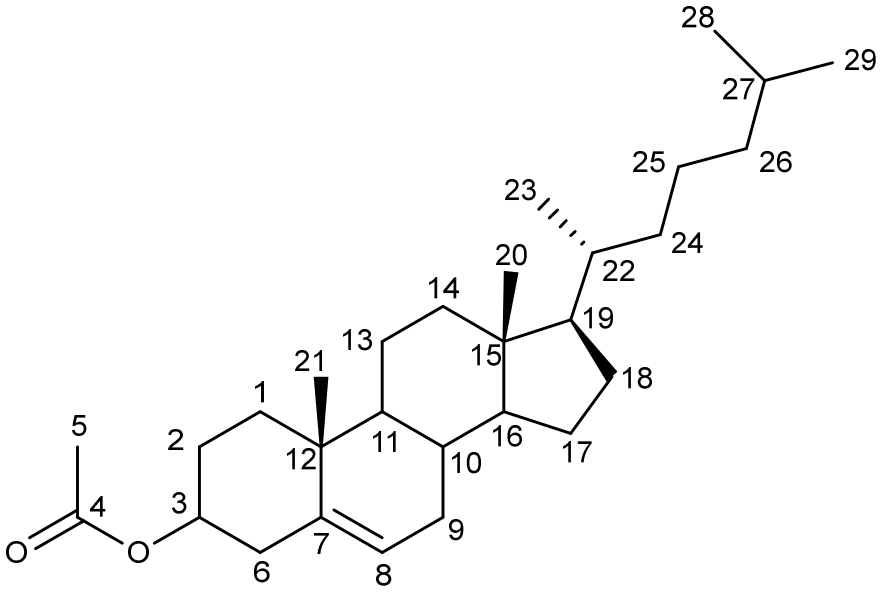
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Figure 2

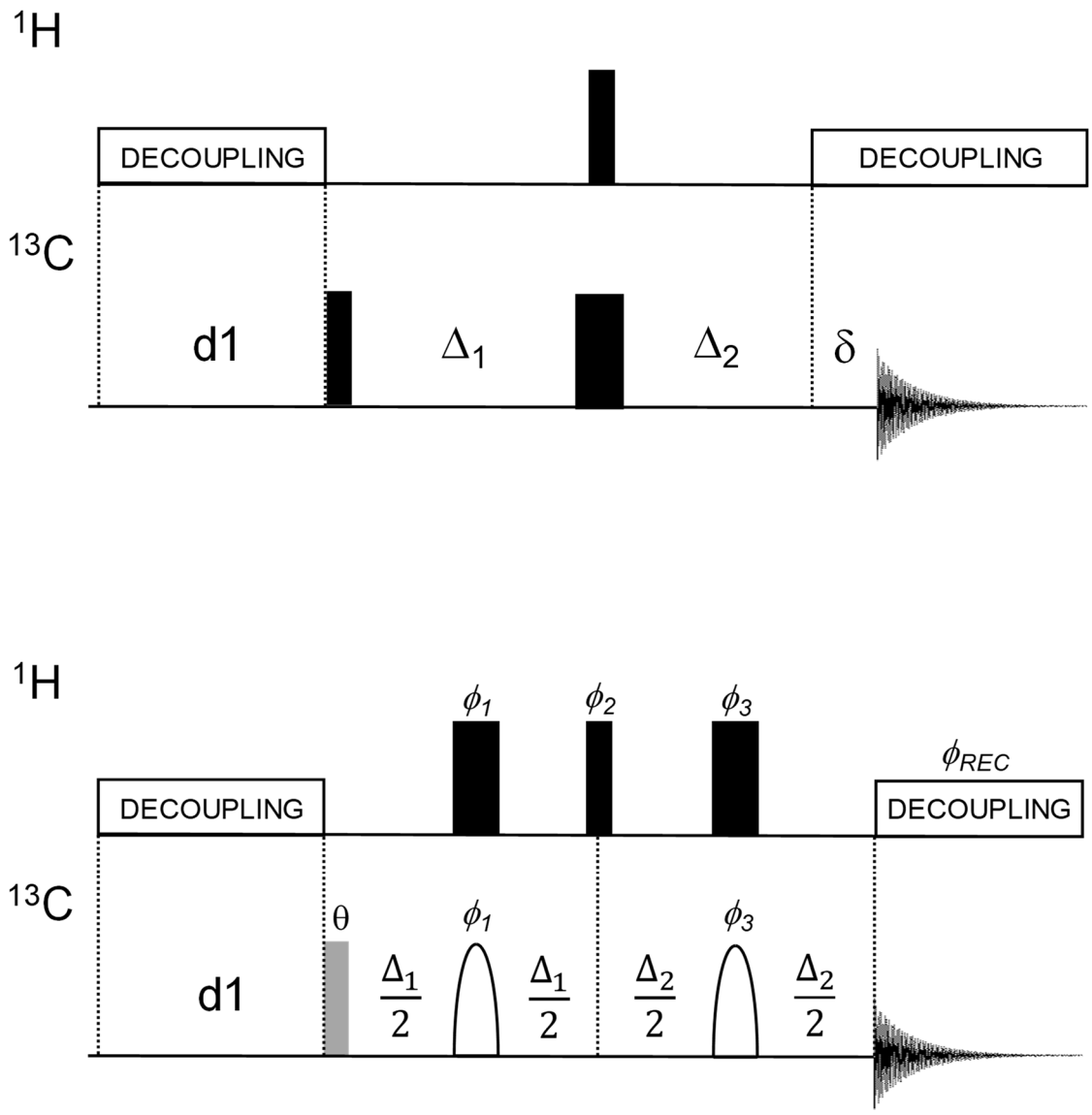
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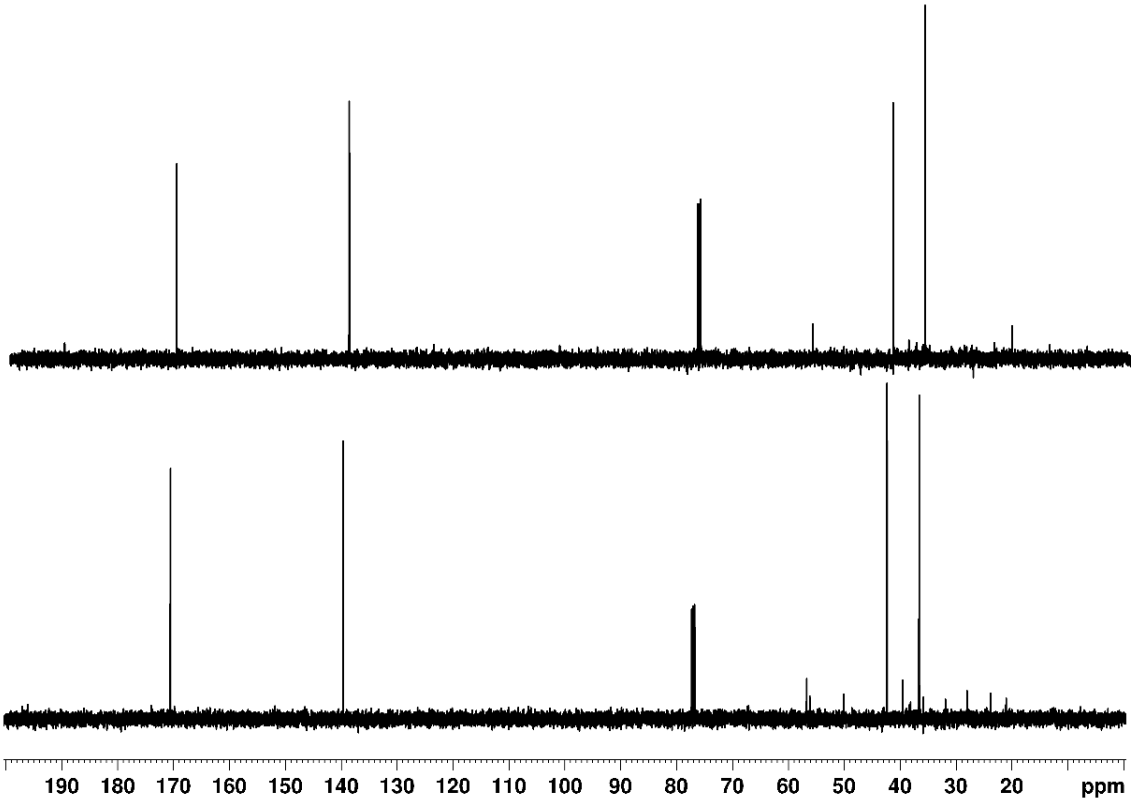
Figure 3



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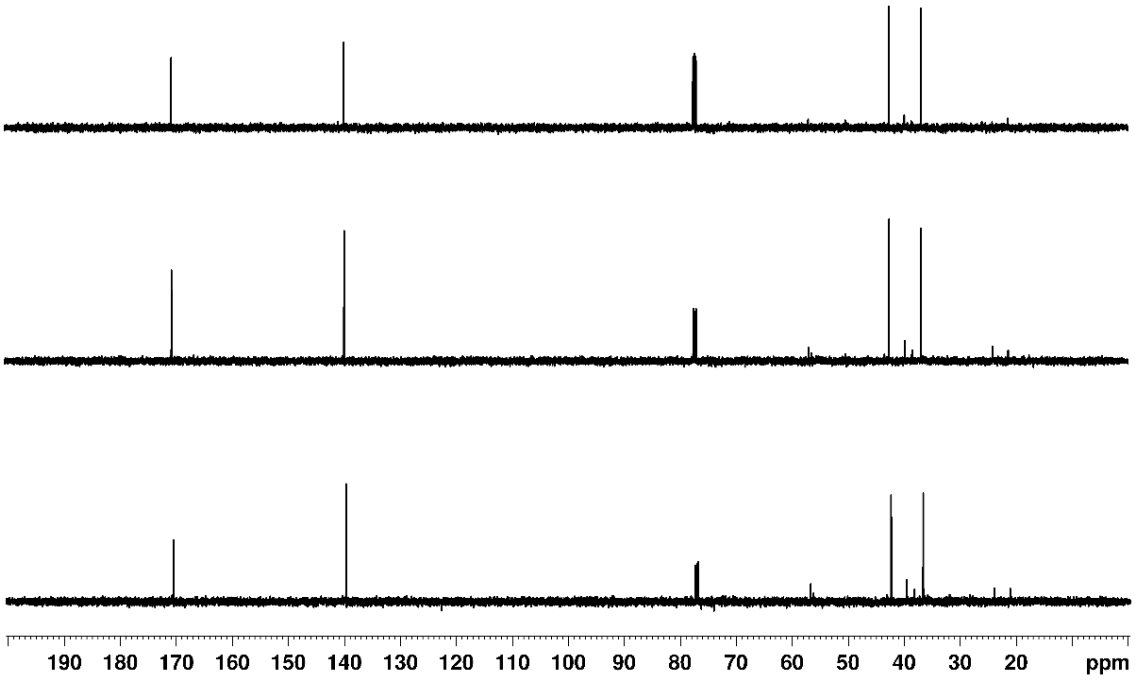

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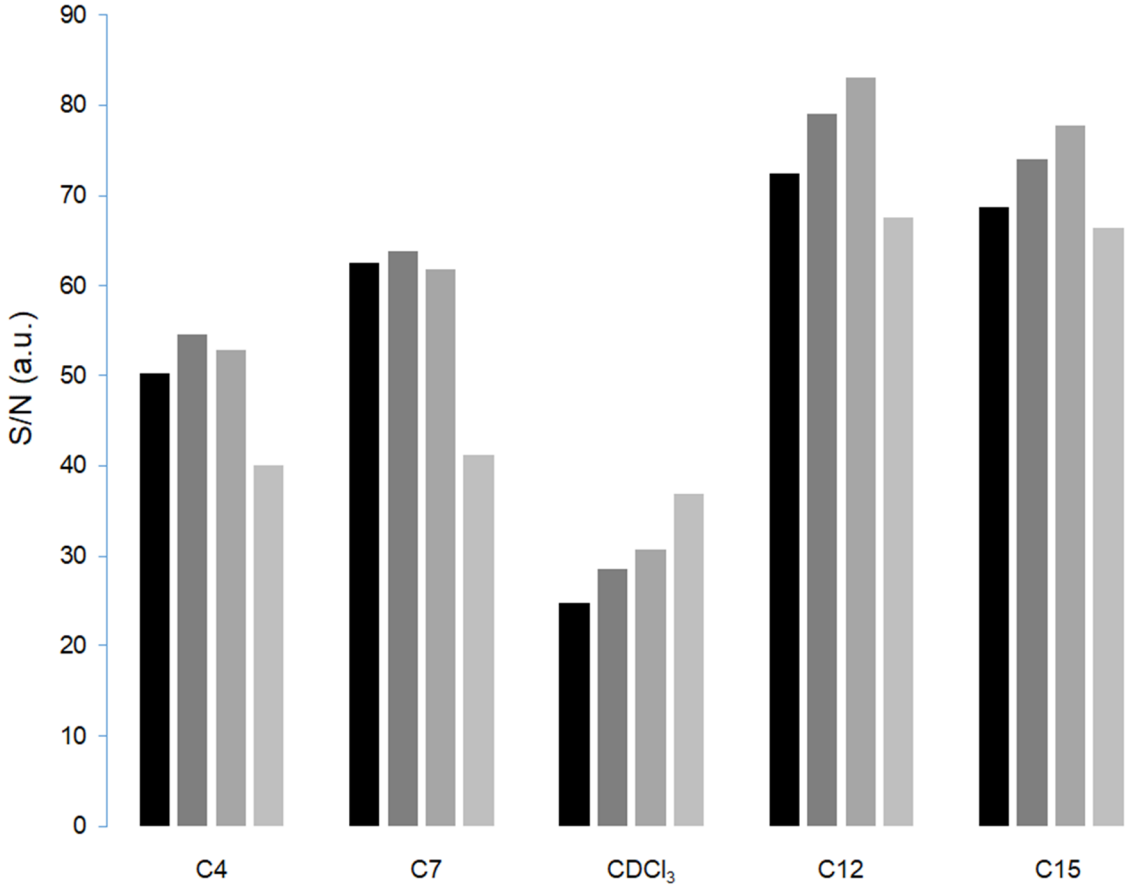

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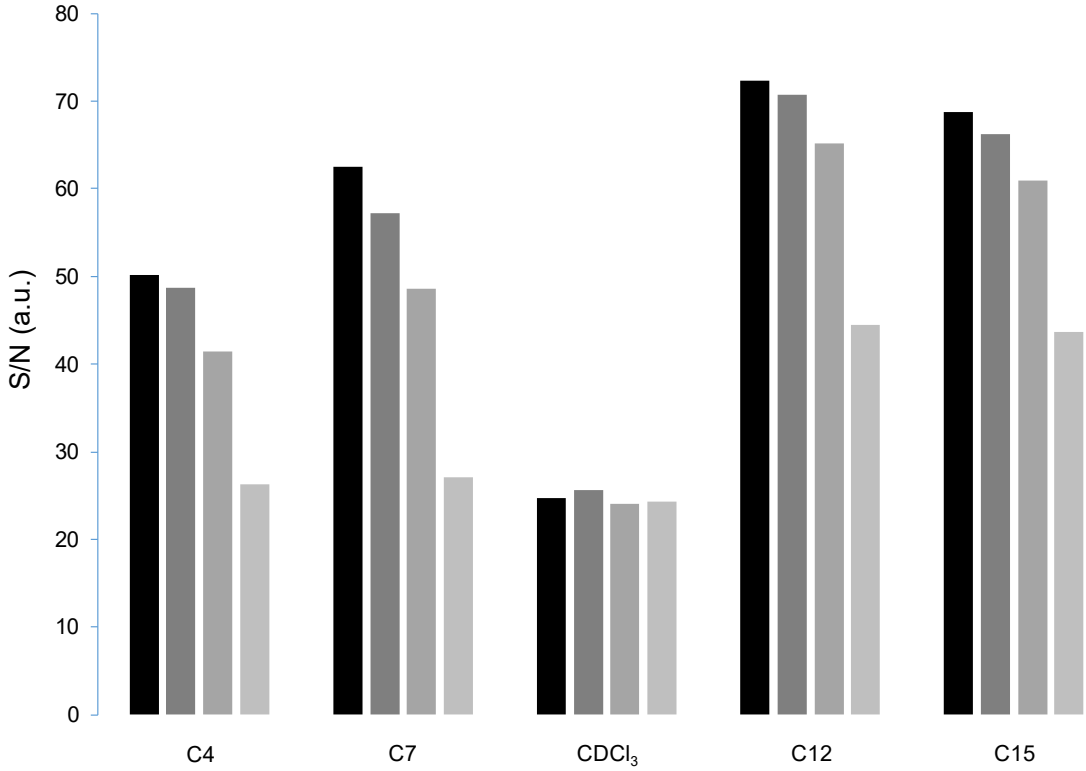

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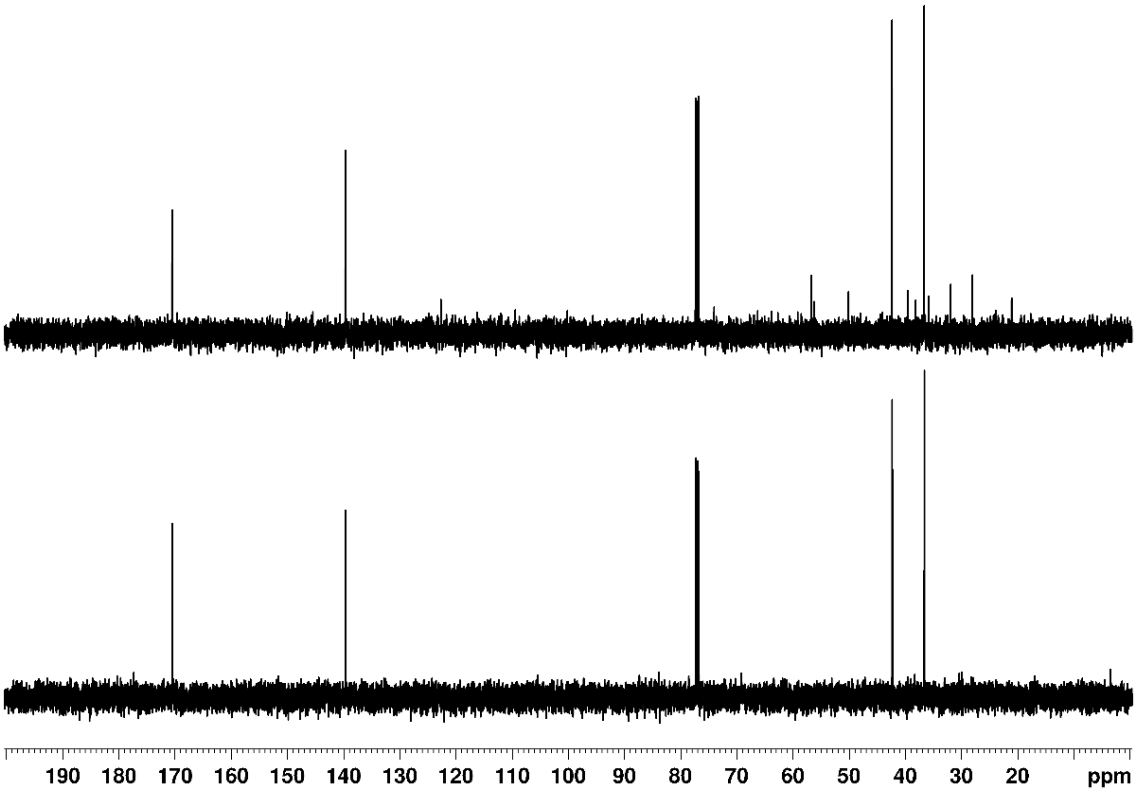
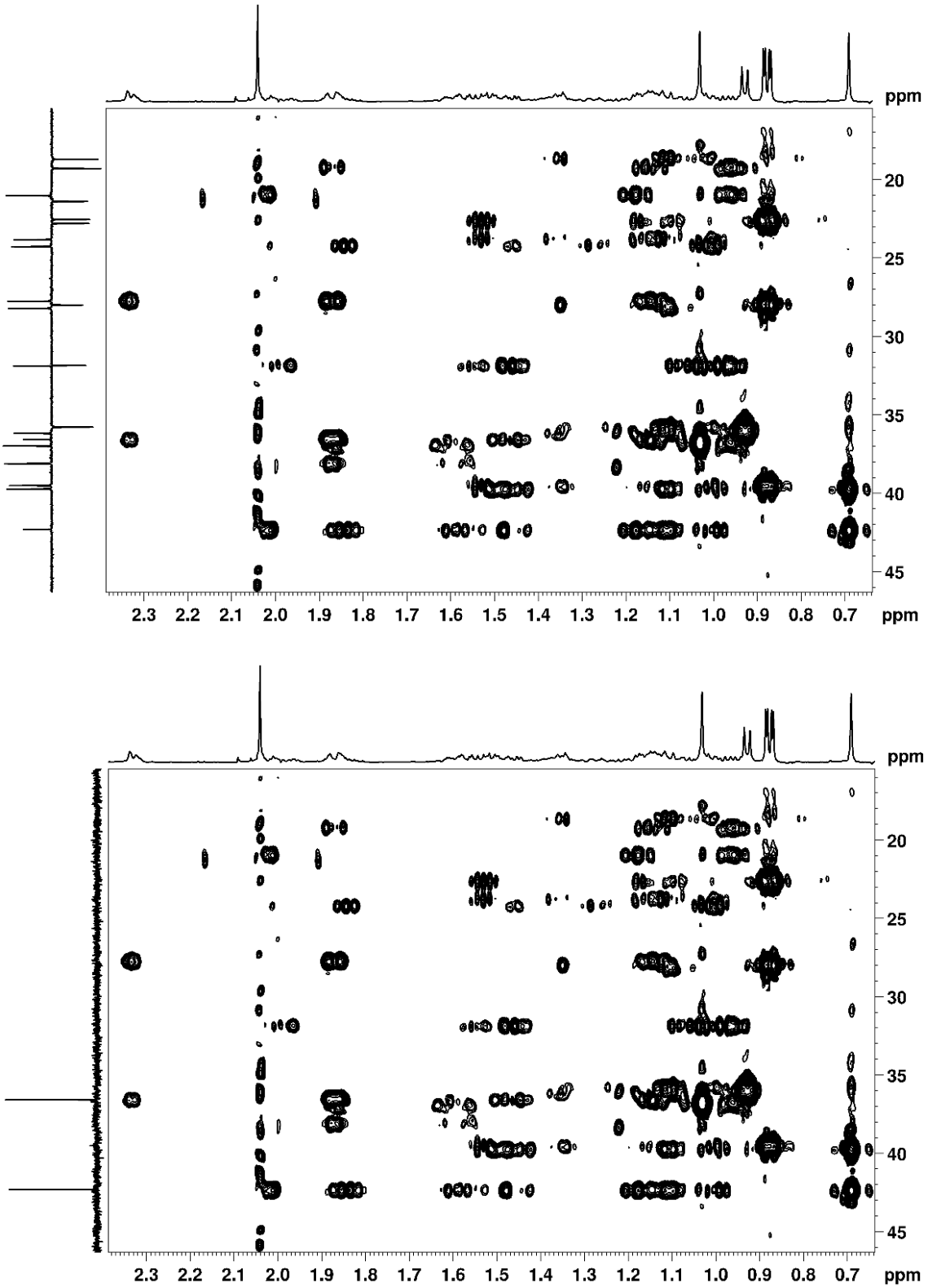


Figure 8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ppm
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ppm

Figure 9

Figure Captions

Figure 1. DEPTQ135 (A) quaternary-carbon-only DEPTQ135 (B), PFG-QCD (C), SEMUT-90 with $\Delta_1 = \Delta_2$ (D), SEMUT-90 with $\Delta_1 \neq \Delta_2$ (E) and SEMUT-GL-90 (F) with ^{1}H-broadband decoupling applied to cholesteryl acetate. 128 scans were acquired. For the DEPTQ135 (A) and the quaternary-carbon-only DEPTQ135 experiments, the relaxation delays d1 and d2 were set to 3 and 1 s, respectively, while the relaxation delay d1 was set to 4 s for the PFG-QCD, SEMUT-90, and SEMUT-GL-90 experiments. The one-bond coupling constant delays Δ_1 were adjusted to a J value of 145 Hz for DEPTQ, PFG-QCD, and SEMUT-90 with $\Delta_1 = \Delta_2$, to $\Delta_1 = J_{min} + 0.146(J_{max}-J_{min})$, $\Delta_2 = J_{max} - 0.146(J_{max}+J_{min})$ for SEMUT-90 with $\Delta_1 \neq \Delta_2$ and to $\Delta_1 = J_{min} + 0.07(J_{max}-J_{min})$, $\Delta_2 = 0.5(J_{max}+J_{min})$, $\Delta_3 = J_{max} - 0.07(J_{max}+J_{min})$, with J_{min} = 120 Hz, J_{max} = 170 Hz.[26, 37] Gradients were applied in the ratio 31:31:31 for the DEPTQ135 experiment and in the ratio 31:31:11 for the quaternary-carbon-only DEPTQ135. The quaternary carbons are labeled following the numbering given in Figure 2.

Figure 2. Cholesteryl acetate carbon numbering.

Figure 3. Original SEMUT-90[12, 26] (top) and proposed *i*QCD pulse sequence for recording quaternary-carbon-only spectra (bottom). In the *i*QCD sequence, the ^{13}C 180° pulses are broadband Burbop-180 pulses (2 ms total duration, 50 kHz sweep width).[30, 31] The first ^{13}C pulse may be adjusted to the Ernst angle[34] for maximizing the sensitivity. The two evolution periods are set to different values: $\Delta_1 = 0.25/(J_{min} + 0.146(J_{max} - J_{min}))$; $\Delta_2 = 0.25/(J_{max} - 0.146(J_{max} - J_{min}))$.[26, 37] δ equals the difference $\Delta_1 - \Delta_2$ to ensure that the acquisition starts at top of the echo. d1 is the relaxation delay. The following phase cycling is applied: $\phi_1 = x, y, -x, -y$; $\phi_2 = 8(y), 8(-y)$; $\phi_3 = 4(-x), 4(-y), 4(x), 4(y)$; $\phi_{rec} = x, -x, x, -x, x, -x, x, -x$. Pulses without labeling are applied along the *x*-axis.

Figure 4. SEMUT-90 (top) and *i*QCD (bottom) spectra of cholesteryl acetate. 64 scans were acquired. The relaxation delay Δ was set to 2 s. The one-bond coupling constant delays were: $\Delta_1 = 0.5/(J_{min} + 0.146(J_{max}-J_{min})) = 3.08$ ms (adjusted to a *J* value of 161 Hz), and $\Delta_2 = 0.5/(J_{max} - 0.146(J_{max}-J_{min})) = 3.92$ ms (adjusted to a *J* value of 128 Hz), with $J_{min} = 120$ Hz, $J_{max} = 170$ Hz. For both experiments, the first pulse θ was adjusted to 90°.

Figure 5. *i*QCD spectra of cholesteryl acetate with the first pulse θ adjusted to 90° (bottom), 60° (middle), and 30° (top). 128 scans were acquired. The relaxation delay d1 was set to 1 s. The one-bond coupling constant delays were: $\Delta_1 = 0.5/(J_{min} + 0.146(J_{max}-J_{min})) = 3.08$ ms (adjusted to a *J* value of 161 Hz), and $\Delta_2 = 0.5/(J_{max} - 0.146(J_{max}-J_{min})) = 3.92$ ms (adjusted to a *J* value of 128 Hz), with $J_{min} = 120$ Hz, $J_{max} = 170$ Hz.

Figure 6. Signal-to-noise ratio measured using the SiNo routine (TopSpin 4.0.8) of C4, C7, CDCl3, C12, C15 in four different *i*QCD spectra with a total experimental time of 22 min: (i) relaxation delay of 4 s, first ^{13}C pulse 90° and 256 scans (black), (ii) relaxation delay of 3 s, first ^{13}C pulse 75° and 320 scans (dark grey), (iii) relaxation delay of 2 s, first ^{13}C pulse 60° and 416 scans (grey), and (iv) relaxation delay of 1 s, first ^{13}C pulse 30° and 592 scans (light grey).

Figure 7. Signal-to-noise ratio measured using the SiNo routine (TopSpin 4.0.8) of C4, C7, CDCl3, C12, C15 in four different *i*QCD spectra recorded with 256 scans: (i) relaxation delay of 4 s, first ^{13}C pulse 90° and total experimental time of 22 min (black), (ii) relaxation delay of 3 s, first ^{13}C pulse 75° and total experimental time of ca. 18 min (dark grey), (iii) relaxation delay of 2 s, first ^{13}C pulse 60° and total experimental time of ca. 13.5 min (grey), and (iv) relaxation delay of 1 s, first ^{13}C pulse 30° and total experimental time of ca. 9.5 min (light grey).

Figure 8. *i*QCD spectra of cholesteryl acetate with the first pulse θ adjusted to 90°. Bottom: pulse calibrated. Top: ^{1}H and ^{13}C pulses lengthened by +20%. 64 scans were acquired. The relaxation delay d1 was set to 4 s. The one-bond coupling constant delays were: $\Delta_1 = 0.5/(J_{min} + 0.146(J_{max}-J_{min})) = 3.08$ ms (adjusted to a J value of 161 Hz), and $\Delta_2 = 0.5/(J_{max} - 0.146(J_{max}-J_{min})) = 3.92$ ms (adjusted to a J value of 128 Hz), with J_{min} = 120 Hz, J_{max} = 170 Hz.

Figure 9. Part of the 7 Hz adjusted HMBC spectrum of cholesteryl acetate. Top: DEPTQ spectrum set as external F_1 projection. Bottom: *i*QCD spectrum set as external F_1 projection.